The Examiner found the assertion that Figure 1 of the specification demonstrates synergistic activity was not convincing, "since the data in Figure 1 herein is unclear as to how the graphical presentation herein may be taken to demonstrate unexpected and synergistic effect in the instant invention." Applicants respectfully disagree and offer the following clarification. Example 8, pages 19 - 20, of the specification describes experiments to determine the effect of lamivudine/PMEA combination on HBV content of human hepatoblastoma cells, which constitutively produce infectious HBV. Fractional inhibitory concentrations (FIC) were calculated for each combination and plotted using the isobologram representation. Figure 1 depicts an isobologram showing a statistically significant synergism. The isobologram was presented according to Berenbaum, M.C. (1985) J. Theor. Biol. 114, 413-431, cited in the specification at page 20. A copy of Berenbaum is attached for the Examiner's convenience. The isobologram representation is a widely accepted method of displaying combination drug effects. Data points falling below the line joining the x and y axis (the zero interaction line), indicate that the two drugs are synergistic in their effect. Data points falling above the line indicate an antagonistic effect. Data points falling on the line indicate additivity.

The demonstration of the synergistic effect of the combination of the present invention is the demonstration of an unexpected effect. Therefore, since the combination of the present invention produces synergistic results, it is not obvious in view of Korba and Glazier alone or in combination. Applicants respectfully request withdrawal of the rejection of claims 1, 2, 4 - 10 , 12 - 15 and 22 under 35 U.S.C. § 103(a).

In view of the foregoing discussion, it is respectfully submitted that the present application is in condition for allowance. An early consideration and notice of allowance are earnestly solicited.

Respectfully submitted,

Date: September 11, 2002

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The Expected Effect of a Combination of Agents: the General Solution

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Interactions between agents (drugs, carcinogens, physiological stimuli, environmental pollutants, etc.) in producing their effects are of fundamental interest and practical importance in virtually every branch of biology and medicine. A combination of agents is said to show interaction when the magnitude of its effect is greater or smaller than expected, expectation being based on the dose-effect relations of the individual agents in the combination. The crux of the matter is to decide what is expected, and various rules have been proposed to this end (for example, that the expected effect is the sum of the effects of the individual constituents of the combination, or that it is the product of these effects, or that it may be calculated from the law of mass action). These rules are valid for combinations of agents with particular and rather restricted types of dose-effect relations, but they have no general validity.

A general solution to this problem is given here, that enables the effects of non-interactive combinations to be calculated directly from the dose-effect relations of the individual agents (whether expressed algebraically or numerically), regardless of the particular types of dose-effect relations involved. This solution is based on the fact that, when an effect of particular magnitude is produced by a combination of n agents which do not interact to produce that effect, the point representing the combination in the n-dimensional space spanned by the dose-axes of the individual agents lies in the same $(n-1)$-dimensional hyperplane as those representing other combinations iso-effective with it and iso-effective amounts of the individual agents.

Methods for calculating the effect of a non-interactive combination as the sum or product of the effects of its constituents, or from the law of mass action, each of which is correct in appropriate cases, may be deduced (without invoking mechanisms of action) by applying this general principle to particular types of dose-effect relations.

Introduction

A recurring problem in biology and medicine is that of calculating the expected quantitative effect of a combination of agents simply from information about their individual dose-effect relations, and without reference to

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their mechanisms of action, which may be insufficiently understood (Bliss, 1939; Plackett & Hewlett, 1963; Finney, 1942, 1971; Hewlett, 1969; Ashford, 1981). This problem is of general interest, arising for example in studies of antibiotics (Thomas, Leonard & Alford, 1976; Berenbaum, 1978; Klastersky & Zinner, 1982; Berenbaum, Yu & Felegie, 1983), cancer chemotherapeutic drugs (Werkheiser *et al.*, 1973; Berenbaum, 1981; Teller *et al.*, 1982; Benz *et al.*, 1983), carcinogens (Rothman & Keller, 1972; Selikoff & Hammond, 1975; Doll, 1977; Reif & Colton, 1984), agents causing chromosomal aberrations (Livingston & Dethlefsen, 1979; Morgan & Cleaver, 1982), immunosuppressive agents (Berenbaum, 1977), inducers of hepatic and renal enzymes (Sunderman, Bibeau & Reid, 1983), inducers of lipid peroxidation (Harvey & Klaasen, 1983), inhibitors of mitochondrial respiration (Fry & Williams, 1984), ionizing and non-ionizing radiations (Dewey *et al.*, 1971; Han & Elkind, 1978; Dewey, 1979; Scott, 1984; Christensen, Wahl & Smedshammer, 1984), insecticides and herbicides (Bliss, 1939; Hewlett, 1969; Wilkinson, 1976; Stratton, 1983; Chou & Talalay, 1984), drugs affecting neuronal function (Yeung & Rudy, 1980; Griffiths *et al.*, 1983), neurophysiological stimuli (Hyman & Frank, 1980), drugs acting on smooth muscle (Campbell *et al.*, 1977; Pöch & Holzmann, 1980; Kreisman *et al.*, 1981) and environmental pollutants (Francis & Petersen, 1983*a*,*b*; Mustafa *et al.*, 1984; Porter *et al.*, 1984). This problem also arises in hypotheses about the interactions of environmental agents in causing disease (Rothman, 1976; Walter, 1983; Walter & Holford, 1978; Berenbaum, 1985).

It is important to be able to decide whether the observed effect of a combination is of the expected magnitude (zero interaction) or whether the agents have interacted, for instance, by altering each other's metabolism or excretion, inhibiting repair mechanisms, or blocking alternative metabolic pathways, to produce an effect greater or smaller than expected (synergy or antagonism, respectively). Synergistic interactions have implications of gain (for example, therapeutic, as in the treatment of disease with drugs—Thomas *et al.* (1976), Campbell *et al.* (1977), Berenbaum (1981), Klastersky & Zinner (1982), Berenbaum *et al.* (1983); or economic, as in the large-scale use of pesticides—Wilkinson (1976)), and the reverse holds for antagonistic interactions. Synergy raises the possibility of substantially modifying an effect without necessarily altering the levels of all the agents responsible for it. For example, with synergistically acting environmental carcinogens, a greater effect on cancer incidence might result from a sharp reduction in the level of one easily controlled carcinogen than from lesser (and less easily achieved) reductions in the levels of several of them (Selikoff & Hammond, 1975; Doll, 1977). Further, the demonstration that a significant

interaction exists may itself throw light on the agents' modes of action and suggest directions for further investigation.

We are not concerned here with the alternative approach of mathematically modelling the effects of agents on biological systems, i.e. calculating effects from knowledge of or hypotheses about underlying mechanisms (Webb, 1963; Ariëns, Simonis & van Rossum, 1964; Ashford & Cobby, 1974; Chou & Talalay, 1977, 1981, 1984). This approach is most useful in simple systems where the main parameters are known and measurable (as in the effects of inhibitors on enzymes in solution). Its use in more complex situations of biological or medical interest may be limited when important parameters are unknown or inaccessible to measurement. These limitations often lead to construction of a multiplicity of models, varying in assumptions as to underlying mechanisms, and the "expected" effect then depends on the set of assumptions chosen. Even when a model adequately accounts for the effect of a combination of agents, the question still remains as to whether the combination shows zero interaction, synergy or antagonism (Werkheiser *et al.*, 1973).

The approach in this paper is to regard only "input" (amounts of agents) and "output" (effects) as accessible, and the intervening system as a "black box". The penalty is that effects can then be predicted only on the assumption of zero interaction between agents, but they can be predicted as confidently and accurately as the measurements permit, irrespective of the complexities of the system, and any deviation from expectation unequivocally indicates an interaction and its type. Such estimates incidentally provide a useful test for mechanism-based mathematical models in which interactions of the sorts described above are assumed to be absent or negligible, for instance, models of combinations of mutually exclusive enzyme inhibitors (Chou & Talalay, 1977, 1984) or of drugs acting on the same receptors in the central nervous system (Ashford & Cobby, 1974; Ashford, 1981).

A comment on terminology is required. Non-interactive combinations are often said to show additivism. This usage is confusing because it is often taken to mean that the effects of such combinations may be obtained by adding the effects of their constituents. As will be made clear, this is true only in particular circumstances, and the term will be used here to mean zero interaction, without implying anything about relations between the effect of a combination and those of its constituents.

Particular Solutions

Several different methods for estimating the effect of an additive combination are in use at present. Three of these can be shown to be valid in

particular sets of circumstances. Others, which have highly restricted or no demonstrable validity, have been examined elsewhere (Berenbaum, 1981, 1984). The valid methods are as follows, denoting the individual agents in the combination by $x_i (i = 1, 2, \ldots, n)$, the combination by $x_{1,2,\ldots,n}$ and the effects of the constituents by $E(x_i)$.

SUMMATION

The effect of a non-interactive combination is simply the sum of the effects of its constituents, or

$$E(x_{1,2,\ldots,n}) = \sum_{i=1}^{n} E(x_i). \quad (1)$$

This assumption is stated explicitly by many investigators (Rothman & Keller, 1972; Livingston & Dethlefsen, 1979; Morgan & Cleaver, 1982; Sunderman *et al.*, 1983; Griffiths *et al.*, 1983; Mustafa *et al.*, 1984). Many others make it by implication by using statistical methods based on analysis of variance and 2^n-factorial experiments (Campbell *et al.*, 1977; Kreisman *et al.*, 1981; Porter *et al.*, 1984), for determination of interactions in these depends on the same assumption. It is shown below (Appendix 2) that this assumption is valid only when all the agents in the combination show linear dose-effect relations, i.e.

$$E(x_i) = \alpha_i x_i. \quad (2)$$

Such relations may occur, for example, between dose or concentration of an agent on the one hand and, on the other, rate constants of bacterial growth (Garrett *et al.*, 1966) or growth delay of tumours (Crathorn & Roberts, 1965). The summation rule may also be used in the low effect region for many agents (Rothman & Keller, 1972; Scott, 1984), even when the response is non-linear, and in the presence of a predominating background effect due to other agents (as with environmental carcinogens (Guess, Crump & Peto, 1977; Peto, 1978)), for in both cases the deviation of the response from linearity at low doses is generally small and statistically insignificant.

MULTIPLICATION

The effect of the combination is the product of the effects of its constituents, or

$$E(x_{1,2,\ldots,n}) = \prod_{i=1}^{n} E(x_i). \quad (3)$$

The assumption may be stated explicitly in this form (Finney, 1971; Harvey, 1978; Stratton, 1983) or in an equivalent form in which E is expressed as

a fraction of unity and $P = 1 - E$ (Bliss, 1939; Finney, 1942, 1971; Webb, 1963). For the two-agent case, this is

$$P(x_{1,2}) = P(x_1) + P(x_2) - P(x_1) \cdot P(x_2).$$

The multiplicative assumption also underlies the method much used by radiobiologists in which, when X and Y do not interact and a fixed dose of X is added to various doses of Y, it is expected that the whole dose-effect curve of Y will be shifted a fixed distance vertically on a logarithmic scale (Dewey *et al.*, 1971; Han & Elkind, 1978; Dewey, 1979).

The multiplication rule was originally derived from probability theory (Trevan, 1927; Bliss, 1939; Mather, 1940; Finney, 1942, 1971). Just as events A and B are defined as being statistically independent if the probability of their joint occurrence is the product of the probabilities of their individual occurrences, so it is assumed that agents A and B act independently, i.e. do not interact, if their joint and individual effects obey an analogous rule. However, it can be shown (Appendix 2) that this rule is valid only when all the agents in the combination have simple exponential dose-response curves, i.e.

$$E(x_i) = \exp(\alpha_i x_i). \tag{4}$$

This is not surprising. Curves of this sort are generated when discrete targets are inactivated by random single "hits" by discrete quanta of agent, such as may occur with ionizing radiations or DNA-crosslinking agents (Crathorn & Roberts, 1965; Dewey *et al.*, 1971; Erickson *et al.*, 1981). The behaviour of such agents is well described by classic target theory (Crowther, 1924; Turner, 1975), which is itself an application of probability theory.

THE MEDIAN EFFECT PRINCIPLE

When dose-effect relations follow a mass-action law, viz

$$\frac{E(x_i)}{1 - E(x_i)} = \left[\frac{x_i}{M_i}\right]^m \tag{5}$$

where E is the fractional effect, M the dose required for the median (50%) effect and m is the order of the reaction, giving the degree of sigmoidicity of the dose-effect curve, then re-arrangement of the equation derived by Chou & Talalay (1981) for n mutually exclusive inhibitors gives

$$E(x_{1,2,\ldots,n}) = \frac{\left[\sum_{i=1}^{n} \frac{x_i}{M_i}\right]^m}{1 + \left[\sum_{i=1}^{n} \frac{x_i}{M_i}\right]^m}. \tag{6}$$

Chou & Talalay (1977, 1981, 1984) have pointed out that a wide variety of phenomena are governed by the mass-action law and have shown that, not only the Michaelis–Menten equation for enzyme inhibition, but also the Langmuir adsorption isotherm equation, the Henderson–Hasselbach equation relating pH and dissociation, and equations for drug binding to cell receptors may all be put into an equivalent form. They have therefore suggested that equation (6) may describe the behaviour of zero-interacting combinations of agents in all these, and possibly other, situations (Teller *et al.*, 1982). Equation (6) is written in terms of the parameters of sigmoid curves and its validity is therefore restricted to agents with dose-effect curves of these forms.

A necessary condition for using any of the three valid particular solutions is that all the agents in the combination show similar dose-effect relations of the appropriate type, but this condition alone may be insufficient. For example, even when all the agents have simple exponential dose-effect curves, the multiplication rule cannot be used if the target population is heterogeneous in sensitivity (Dewey, 1979). None of these solutions is applicable to the many biologically active agents with dose-effect curves that do not fall into the above categories, for example, exponential curves with shoulders, quadratic curves, curves which are sums or products of more than one function, and so on, and they cannot be used for combinations of agents with different types of dose-effect relations.

These restrictions are not generally appreciated and these methods are consequently often wrongly used, i.e. when dose-response relations are unknown or are of inappropriate type. Effect-summation has incorrectly been used with demonstrably non-linear dose-response curves (Hyman & Frank, 1980; Morgan & Cleaver, 1982; Griffiths *et al.*, 1983) and effect-multiplication when effect is not a simple exponential function of dose (Webb, 1963; Dewey *et al.*, 1971; Rothman, 1976; Han & Elkind, 1978; Harvey, 1978; Stratton, 1983).

The General Solution

I show here that, if the relations between dose and effect of the individual agents in a combination are known over adequate ranges, the effect of a zero-interacting combination can be calculated. The type of dose-effect relation is immaterial; it may be the same or different for the various agents in the combination, and there is no requirement that the relations be expressible as simple algebraic functions, as in the cases described above. The clue is given by the properties of isoboles (Fraser, 1872; Loewe & Muischnek, 1926; Loewe, 1953; Berenbaum, 1977, 1978, 1981), i.e. iso-effect

curves or surfaces in the space spanned by the n axes representing the doses of the n individual agents. It can be shown (Appendix 1) that, if the agents in a combination do not interact in producing the effect of the combination then, irrespective of dose-effect relations, the isobole for that effect satisfies the equation

$$\sum_{i=1}^{n} \frac{x_i}{X_i} = 1 \tag{7}$$

where the x_i's are doses (or concentrations) of the individual agents in a combination and the X_i's the doses (or concentrations) of the agents that individually would produce the same magnitude of effect as the combination. This equation describes an $(n-1)$-dimensional hyperplane which, when $n=2$ or 3, is a straight line or flat plane (Berenbaum, 1981), and thus equation (7) states what may be termed the hyperplane theorem.

(For agents that do not themselves produce the qualitative effect of the combination but may increase or decrease the effect produced by other agents in the combination, X_i in equation (7) may be assumed to be infinite, so that x_i/X_i is zero for such agents, irrespective of their dosage. Thus, equation (7) covers combinations of active with inert agents and of agonists with antagonists as well as combinations of active agents.)

When agents interact to produce an effect, the expression in equation (7) is less than 1 in the case of synergy and greater than 1 for antagonism. When $n=2$ or 3, these interactions may be represented graphically by the familiar concave-up or concave-down isoboles (experimentally determined examples of these are illustrated by Fraser (1872), Werkheiser *et al.* (1973), Thomas *et al.* (1976), Berenbaum (1977, 1981), Yeung & Rudy (1980), Benz *et al.* (1983), Fry & Williams (1984) and Christensen *et al.* (1984)).

NUMERICAL SOLUTIONS

The hyperplane theorem makes it possible to find the effect of a combination of non-interacting agents, whether their dose-effect relations are similar or not, by simple numerical methods, as follows.

All the X_i's have the same effect, that of the combination in question, so we have

$$E(x_{1,2,\ldots,n}) = f_i(X_i) = f_j(X_j) \quad \text{for all } i, j = 1, 2, \ldots, n \tag{8}$$

where the f's are functions giving relation between dose and effect. The set of equations (8) implies that, in a graph of the dose-effect curves of the different agents, the horizontal axis at the level of the effect of the combination, $E(x_{1,2,\ldots,n})$, intersects the various curves at points corresponding to

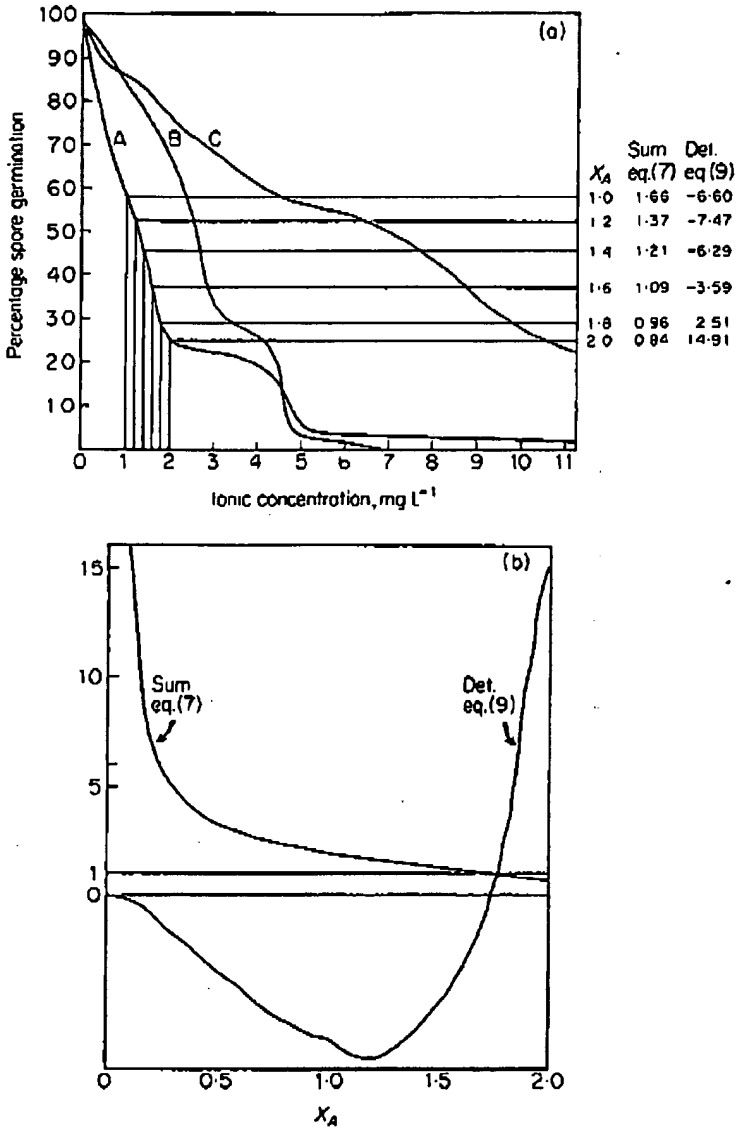

FIG. 1. Numerical methods for finding the effect of a combination of non-interacting agents. (a) Inhibition of spore germination in *Onoclea sensibilis* by (A) Cu^{++}, (B) Cd^{++} and (C) Zn^{++} (Francis & Petersen, 1983*a*). The combination tested contained 1 mg l^{-1} each of Cu^{++}, Cd^{++} and Zn^{++} (Francis & Petersen, 1983*b*) so $x_1 = x_2 = x_3 = 1$. Thus, equation (7) becomes $1/X_A + 1/X_B + 1/X_C = 1$ and equation (9) becomes

$$\begin{vmatrix} X_1 & -X_2 & 0 \\ X_1 & 0 & -X_3 \\ X_1 - 1 & -1 & -1 \end{vmatrix} = 0.$$

Horizontal iso-effect lines are drawn for various trial values of X_A. Their intercepts with the dose-effect curves give trial values for X_A, X_B and X_C, and the resulting values for the sum in equation (7) and the determinant in equation (9) are indicated. These equations are satisfied

their respective X_i's. The level of $E(x_{1,2,\ldots,n})$ can therefore be found by substituting in equation (7) the given values of $x_1, x_2, \ldots, x_n$ and trial iso-effective values of the X_i's read off the dose-effect curves. Values of the X_i's satisfying equation (7) indicate the level of the effect of the combination. The procedure is illustrated in Fig. 1(a) and (b).

Alternatively, we may use the fact that the point $(x_1, x_2, \ldots, x_n)$ representing the combination and those representing the X_i's lie in the same hyperplane in $\mathcal{R}^n$. The X_i's are necessarily non-zero, so a necessary and sufficient condition for coplanarity of the points is that the determinant

$$\begin{vmatrix} X_1 & -X_2 & 0 & \cdots & 0 \\ X_1 & 0 & -X_3 & \cdots & 0 \\ \vdots & \vdots & \vdots & & \vdots \\ X_1 & 0 & 0 & \cdots & -X_n \\ X_1 - x_1 & -x_2 & -x_3 & \cdots & -x_n \end{vmatrix} = 0. \qquad (9)$$

(Equations (7) and (9) are equivalent.) Thus, values of this determinant are calculated for various trial iso-effective values of the X_i's, corresponding to various levels of effect, as read off the dose-response curves. The required hyperplane lies in an interval of effect in which the sign of the determinant changes, and may be identified as accurately as the data permit by reducing the interval. The procedure is illustrated in Fig. 1(a) and (b).

When $n = 2$ and the dose-effect curves for both agents are known over the relevant ranges, there is a particularly simple graphic method for finding the iso-effect hyperplane in which a zero-interacting combination (x_1, x_2) lies, as here the hyperplane is a straight line. The whole interaction can be depicted by drawing the straight-line isoboles for the measured effects on a two-dimensional graph and it is then a trivial task to identify the isobole that passes through the point with coordinates (x_1, x_2), using interpolation between measured effects if necessary. This isobole gives $E(x_{1,2})$ directly.

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when X_A lies between 1·6 and 1·8. (b) Close limits for X_A may be obtained by plotting the left-hand sides of equations (7) or (9) against trial values of X_A. The equations are satisfied when $X_A \sim 1{\cdot}74$ (and $X_B \sim 3{\cdot}1$, $X_C \sim 9{\cdot}5$). Plotting the determinant provides the closer limits. Thus, the expected effect of the combination is that of 1·74 mg l^{-1} of Cu^{++} (or of 3·1 mg l^{-1} Cd^{++} or 9·5 mg l^{-1} Zn^{++}), i.e. 33% spore germination, as determined from the dose-effect curves in (a). This is the effect expected assuming no interactive effects on spore germination between Cu^{++}, Cd^{++} and Zn^{++}. The observed effect was 4% spore germination (Francis & Petersen, 1983*b*), showing that the combination was synergistic. Note that determination of complete dose-effect curves may not be necessary. Relatively few measurements could have shown that equations (7) and (9) were satisfied at Cu^{++} levels between 1·5 and 2 mg l^{-1}, Cd^{++} between 2·5 and 3·5 mg l^{-1} and Zn^{++} between 8 and 10 mg l^{-1}. Thus, the expected effect of the combination (from (a)) lay between 30% and 50% spore germination, and the observed value of 4% indicated synergy.

ALGEBRAIC SOLUTIONS

With dose-effect relations that have simple algebraic forms, the hyperplane theorem may enable the effects of combinations to be expressed algebraically. From equation (7) it follows that

$$X_i = x_i\left[1 - \sum_{\substack{j=1 \\ j\neq i}}^{n} \frac{x_j}{X_j}\right]^{-1}. \quad (10)$$

When the functions in equation (8) are strictly monotonic and therefore invertible, we may write

$$f_j^{-1}(f_i(X_i)) = X_j \quad (11)$$

and thus

$$X_i = x_i\left[1 - \sum_{\substack{j=1 \\ j\neq i}}^{n} \frac{x_j}{f_j^{-1}(f_i(X_i))}\right]^{-1}. \quad (12)$$

The predicted effect of the combination is then simply $f_i(X_i)$. The generality of equation (12) may be illustrated by using it to derive equations (1), (3) and (6) for the effects of combinations of agents directly from their respective dose-effect equations (2), (4) and (5) as follows.

For combinations in which the effect of each agent is given by

$$E(x_i) = f_i(x_i) = g(\alpha_i x_i) \quad (13)$$

where g is a monotonic function (the same for all agents in the combination) and α_i a constant depending on the agent then, for iso-effective X_i and X_j,

$$g(\alpha_i X_i) = g(\alpha_j X_j) \quad \text{so } X_j = \frac{\alpha_i}{\alpha_j} X_i.$$

As $X_j = f_j^{-1}(f_i(X_i))$, substitution in equation (12) gives

$$X_i = x_i\left[1 - \sum_{\substack{j=1 \\ j\neq i}}^{n} \frac{\alpha_j x_j}{\alpha_i X_i}\right]^{-1}$$

which, on rearrangement, gives

$$X_i = \frac{1}{\alpha_i} \sum_{i=1}^{n} \alpha_j x_j.$$

So

$$E(x_{1,2,\ldots,n}) = f_i\left[\frac{1}{\alpha_i} \sum_{i=1}^{n} \alpha_j x_j\right] = g \sum_{i=1}^{n} \alpha_i x_i \quad (14)$$

that is, the effect of a zero-interactive combination is found simply by substituting $\sum_{i=1}^{n} \alpha_i x_i$ for $\alpha_i x_i$ in equation (13).

The three cases described above, for which valid rules are in use for calculating the effect of a zero-interactive combination, all fall into this category, for g is the identity function in the summative case and the exponential function in the multiplicative case and, in the mass action case, rearrangement of equation (5), with $\alpha_i = M_i^{-1}$, gives

$$g(\alpha_i x_i) = \frac{(\alpha_i x_i)^m}{1+(\alpha_i x_i)^m}. \tag{15}$$

In the summative case, substitution of $\sum_{i=1}^{n} \alpha_i x_i$ for $\alpha_i x_i$ in equation (2) immediately gives $E(x_{1,2,\ldots,n}) = \sum_{i=1}^{n} \alpha_i x_i = \sum_{i=1}^{n} E(x_i)$, which is equation (1). In the multiplicative case, substitution in equation (4) gives

$$E(x_{1,2,\ldots,n}) = \exp\left[\sum_{i=1}^{n} \alpha_i x_i\right] = \prod_{i=1}^{n} \exp(\alpha_i x_i)$$

$$= \prod_{i=1}^{n} E(x_i),$$

which is equation (3).

In the mass-action case, substitution in equation (15) gives equation (6) directly.

When g is an isomorphism from the set $\{(\alpha_i x_i), +\}$ to the set $\{g(\alpha_i x_i), *\}$, where $*$ is a binary operation (as in the first two cases, where the operation is addition or multiplication, respectively), then $*$ gives the rule for determining the effect of a zero-interactive combination directly from the effects of its constituents, i.e.

$$E(x_{1,2,\ldots,n}) = E(x_1) * E(x_2) * \ldots * E(x_n).$$

Thus, these three cases are particular solutions of the general equation (12). It should be noted that equation (14) allowed the rules for calculating the effects of zero-interactive combinations in these cases to be derived directly from the dose-effect equations of the individual agents, without reference to mechanisms of action. What makes this possible is that, as equation (13) implies, the dose-effect curves of all the agents in the combination are similar (i.e. superimposable by linear scaling of the dose-axes). Clearly, the utility of equation (14) is not limited to these three cases; it is applicable to any combinations meeting the requirements of equation (13).

For a sufficiently complicated example illustrating this point, consider Ashford's (1981) equation for the effect of an agent that behaves according to the law of mass action. Changing the symbols to those used in this paper,

this is

$$E(x_i) = h\left\{\log\left(\frac{1+k_i\alpha_i x_i}{1+\alpha_i x_i}\right)\right\} + \varepsilon \qquad (16)$$

where h is a "monotonic increasing function of the operative level" of the agent, k_i its intrinsic activity, α_i its association constant and ε an error term which may be disregarded in this context. Equation (16) is clearly a particular case of equation (13), with $\alpha_i x_i$ having the same meaning in each and the rest of the expression in equation (16) representing g. Ashford (1981) says that, if two such drugs are interactive at their common site of action, $k_1 \neq k_2$, so we put $k_1 = k_2 = k$ for the non-interactive case.

Substitution of $\sum_{i=1}^{n} \alpha_i x_i$ for $\alpha_i x_i$ in equation (16) gives, for two agents,

$$E(x_{1,2}) = h\left\{\log\left(\frac{1+k\alpha_1 x_1 + k\alpha_2 x_2}{1+\alpha_1 x_1 + \alpha_2 x_2}\right)\right\}$$

which is the equation that Ashford (1981) derives from mass action considerations for two agents acting non-interactively at one site (with $k_1 = k_2 = k$).

CHOICE OF COMBINATION

Dose-effect relations are necessarily determined only over a restricted range of doses for each agent ($\min_i, \max_i$), where $\min_i$ is usually zero. Calculation of the expected effect of a combination using the methods described here requires that the range for each agent includes its X_i. Equation (7) shows that this is assured, for a zero interactive combination, under the following conditions.

(1) If the maximum (minimum) doses tested of the individual agents are iso-effective, then the x_i's must be chosen so that

$$\sum_{i=1}^{n} \frac{x_i}{\max_i} \leq 1 \quad \text{and} \quad \sum_{i=1}^{n} \frac{x_i}{\min_i} \geq 1.$$

(2) If the maximum (minimum) doses tested are not equally effective, supposing $\max_j$ is the least effective of these (or $\min_j$ the most effective), the x_i's must be chosen so that the above conditions hold with quantities of each ith agent ($i \neq j$) iso-effective with $\max_j(\min_j)$ substituted for each $\max_i(\min_i)$. This has the effect of restricting the region in which the combination may be placed to one in which the maximum (minimum) doses of the agents are iso-effective.

When these conditions are not met, it is not possible to calculate the effect of a zero interactive combination, but testing combinations that breach these conditions is not necessarily uninformative. For example, in a 2^n-

factorial experiment in which all agents are tested at two levels, zero and x_i, separately and combined, the first condition is violated because $\max_i = x_i$ for all agents, so

$$\sum_{i=1}^{n} \frac{x_i}{\max_i} = n > 1.$$

Nevertheless, in these and other experiments in which the x_i's are too large, antagonism may be indicated unequivocally if the observed effect of the combination is less than the minimum effect of any $\max_i$. Conversely, a combination that violates the second inequality (which may occur only when all $\min_i > 0$) is synergistic if its observed effect exceeds the maximum effect of any $\min_i$. When combinations do not satisfy any of the above requirements as to magnitude of x_i's or of effects, it is not possible to say whether they show zero interaction, synergy or antagonism. A large number of published investigations fail in this respect (Berenbaum, 1977, 1981).

NON-MONOTONIC FUNCTIONS

These can be handled in the ways described above for monotonic functions, but care is needed in determining the $f_j^{-1}(f_i(X_i))$'s for equation (12) or the trial X_i's for equations (7) or (9), as particular magnitudes of effect are produced at more than one level of one or more of the agents. It is not difficult to see that the appropriate values (if they exist) are found by matching the successive ascending and descending sections of the dose-response curves of the agents, matching commencing at zero dose. However, except perhaps in the simplest non-monotonic cases, analyses of interactions between such agents may be fraught with uncertainty. For instance, when increasing doses produce increasing effects over some dose-range(s) and decreasing effects over some other(s), it may not be possible to decide whether an effect less than expected represents synergy or antagonism. A different sort of analysis may be required for such agents.

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APPENDIX 1

Investigators have for many years taken linear isoboles (and their algebraic equivalent, equation (7)) as indicating zero-interaction, because "combinations" of an agent with itself are held to show zero-interaction and they show linear isoboles. However, this model can be applied directly only to combinations of agents with dose-effect curves that are either identical or simple linear transforms of each other. It is often assumed that zero-interactive combinations of agents with dissimilar dose-effect curves should also have linear isoboles, but no proof of this appears to have been suggested. Indeed, Löewe (1953), whose analysis of isoboles underlies much of their usage since, doubted whether this was the case. Perhaps because of this, there is no consensus as to the correct criteria for zero-interaction (and thus for synergy or antagonism). A wide variety of methods, including the isobole method, is in use, choice of method often depending more on the whim of the investigator than on demonstrable validity.

It is therefore important to show that the isobole method and equation (7) apply to combinations of all types, irrespective of the nature of the dose-effect relations. It would then follow that methods giving results discordant with these are invalid. The procedure adopted here is (i) we first decide what is meant by zero-interaction in a combination, (ii) we find what parameters of dose-effect relations could be used in a general method for detecting zero-interaction and (iii) we construct a zero-interactive combination of one agent only, and compare it with a combination of different agents to find conditions in which the two are indistinguishable in the parameters found in (ii). In these conditions, both combinations must be zero-interactive.

(i) *What is zero-interaction?* In a sense, most agents "interact" with themselves in producing their effects, in that individual molecules or quanta of agent assist or hinder each other's actions at the target site. Absence of such self-interaction is shown only by agents that act according to classic target theory, with individual quanta randomly inactivating discrete and independent targets. In these circumstances, exponential dose-effect curves are generated, and whenever the dose-effect curve is not of this form, self-interaction of some type may be presumed to exist.

However, such effects are not the issue here, where we deal with an interaction between different agents in a combination which causes its effect to be greater or smaller than that expected from the dose-effect curves of the agents themselves. To illustrate the difference between the two phenomena, suppose agent A shows self-interaction of the sort described above and consider a spurious "combination" of various amounts of A. Clearly, the effect of the combination can only be that which would be expected from the dose-effect curve of A, so the combination shows zero-interaction.

Looking at the matter in another way, interaction in this sense implies some advantage or disadvantage in using, say, A and B together as compared with using them on their own. Now, it would not make sense to assert that, because A interacts with itself (as shown by the shape of its dose-effect curve), there is advantage or disadvantage in using A in combination with A, as compared with using A on its own.

Thus "combinations" consisting of various quantities of only a single agent show zero-interaction, and these form a useful model in finding a criterion for zero-interaction for combinations of different agents, as follows.

(ii) *Parameters for a general zero-interaction criterion.* As shown above, there are a variety of criteria for identifying zero-interacting combinations where these are of agents with particular types of dose-effect relations. However, we seek a criterion that is applicable irrespective of the nature of the dose-effect relations of the individual agents. This criterion must therefore depend on quantitative factors that are perfectly general among agents and their combinations. Dose-effect relations are of almost unlimited variety, but four parameters are always available for any combination, irrespective of the nature of these relations. These are (i) the effects of the individual constituents of the combination, (ii) the effect of the combination, (iii) the amounts of the individual constituents, $x_1, x_2, \ldots, x_n$ and (iv) the amounts, $X_1, X_2, \ldots, X_n$, of the individual agents that, when used separately, would each have the same magnitude of effect as the combination.

We wish to relate a parameter of the constituents ((i) or (iii)) to a parameter of the combination ((ii) or (iv)) and, intuitively, it might be

supposed that (ii) the effect of the combination, could be derived directly from (i), the effects of its constituents. Indeed, this is possible in certain cases, as shown above, where the effect of the combination was either the sum or product of the effects of its constituents. However, combinations for which this is possible are restricted in scope and, in any case, the method of calculation differs for agents with different types of dose-effect relations. Accordingly, no general method is available for such calculations, and certainly not for combinations of agents of different types.

Therefore, if we wish to find a general criterion independent of the types of dose-effect relations, we must seek a relation between (iii) and (iv), that is, the x_1's and the X_1's.

(iii) *Comparison of real and dummy combinations.* Consider combination A, consisting of amounts $x_1, x_2, \ldots, x_n$ of n agents, with effect $E(A)$. Let the amounts of the individual agents iso-effective with A be $X_1, X_2, \ldots, X_n$. We discover whether A is zero-interactive or not by making a dummy combination B that is zero-interactive by construction, and then comparing A and B with respect to the two parameters selected above.

First, choose any of the agents in A (say, agent 1, with iso-effective dose X_1) and make n separate k_i-fold dilutions of this agent, where $k_i = X_i/X_1$. The iso-effective dose of the ith diluted material is X_i, but this dose contains $k_i^{-1}X_i = X_1$ of agent 1, ensuring that each X_i of the diluted materials is still iso-effective with A. (We assume for the purpose of this argument that mere dilution or concentration does not influence effect.)

These procedures give us n materials, the doses of which that are iso-effective with A match those of the agents in A. These may be labelled as if they are different agents but, in fact, they are "dummies" for the real agents in A. However, an investigator given only quantitative information about the doses $X_1, X_2, \ldots, X_n$ and their effects would not be able to distinguish between the real agents and their dummies.

Now take doses $x_1, x_2, \ldots, x_n$ of the dummy agents to make combination B. Each ith constituent of B contains $(X_1/X_i)x_i$ of agent 1, so the total amount of this agent in B is $X_1 \sum_{i=1}^{n} x_i/X_i$. B contains only one agent, so it necessarily shows zero-interaction in the sense used here, that is, its effect cannot be other than that expected from the total amount of agent 1 it contains. Therefore,

$$E(B) = E\left[X_1 \sum_{i=1}^{n} \frac{x_i}{X_i}\right].$$

We do not know the effect of B (we certainly cannot assume that it will equal that of A). However, either $E(B)$ equals $E(A)$ or it does not, and we examine both possibilities.

(a) $E(B) = (A)$ implies that doses of any agents that are iso-effective with B are also iso-effective with A and thus, for effects that are monotonic functions of dose, $X_{iB} = X_{iA}$ (where these are, respectively, the doses of materials used to construct B and A that are also iso-effective with these combinations). Also, $x_{iB} = x_{iA}$ by construction. Thus, A and B are quantitatively alike in constituents (the x_i's), effect and iso-effective doses (the X_i's), and an investigator restricted to this information, as must be the case where a general criterion for zero-interaction is wanted, would not be able to distinguish between A and B. Accordingly, as B is zero-interactive, so also is A. It remains to find the relation between the x_i's and the X_i's for these combinations. $E(B) = E(A)$ implies that

$$E\left[X_1 \sum_{i=1}^{n} \frac{x_i}{X_i}\right] = E(X_1).$$

So, for effects that are monotonic functions of dose,

$$\sum_{i=1}^{n} \frac{x_i}{X_i} = 1, \quad \text{which is equation (7).}$$

Thus, zero-interactive combinations satisfy equation (7).

(b) $E(B) \neq E(A)$. Both combinations are made of identical amounts, $x_1, x_2, \ldots, x_n$, of materials which have identical X_i's (doses iso-effective with A). B is zero-interactive, having the expected effect and therefore, as effect is a single-valued function of dose and A has a different effect from B, the effect of A is not what is expected and thus A does not show zero-interaction, but synergy or antagonism.

Now $E(B) \neq E(A)$ implies that $X_{iB} \neq X_{iA}$. However $x_{iA} = x_{iB}$ as before so, by the same reasoning as before,

$$\sum_{i=1}^{n} \frac{x_i}{X_i} \neq 1.$$

Thus, combinations that are not zero-interactive do not satisfy equation (7).

When $E(A)$ exceeds expectation (synergy), $E(A) > E(B)$ and so $X_{iA} > X_{iB}$. Conversely, when $E(A)$ is less than expected (antagonism), $E(A) < E(B)$ and so $X_{iA} < X_{iB}$. As $x_{iA} = x_{iB}$,

$$\sum_{i=1}^{n} \frac{x_{iA}}{X_{iA}} \begin{cases} < 1 \text{ for synergy} \\ > 1 \text{ for antagonism} \end{cases}$$

When $n = 2$ or 3, the isobolar lines or surfaces generated by these inequalities are concave-up in the case of synergy and concave-down in the case of antagonism.

The foregoing analysis covers only combinations of agents that (a) have monotonic dose-effect curves and (b) can all produce the effect of A, i.e. a value can be assigned to X_i for all $i = 1, 2, \ldots, n$. The argument requires only trivial modification, and is not invalidated, for combinations including agents with non-monotonic curves or that have notionally infinite X_i's. Such combinations have been discussed above.

APPENDIX 2

The proofs that the summative and multiplicative rules hold only when all the agents in a combination show respectively linear or exponential dose-effect relations are elementary. However, as these rules are very widely used in quite inappropriate circumstances, the proofs are given here in the hope of discouraging this improper use.

Summative Rule

Let the effect of a zero-interactive combination $(x_1, x_2, \ldots, x_n)$ be E. Then $\sum_{i=1}^{n} f_i(x_i) = E$ (equation (1)) and $\sum_{i=1}^{n} (x_i/X_i) = 1$ (equation (7)) imply that

$$\sum_{i=1}^{n} f_i(x_i) = \sum_{i=1}^{n} \left(\frac{E}{X_i}\right) x_i.$$

This holds for all x_i's satisfying equation (7), and the only solution is $f_i(x_i) = \alpha_i x_i$ where $\alpha_i = E/X_i$ (a constant) that is, the effect of a zero-interactive combination is the sum of the effects of its constituents only when all agents in the combination show linear dose-effect relations.

Multiplicative Rule

$\prod_{i=1}^{n} f_i(x_i) = E$ (equation (3)) and $\sum_{i=1}^{n} (x_i/X_i) = 1$ (equation (7)) imply that

$$\sum_{i=1}^{n} \ln (f_i(x_i)) = \ln E = \sum_{i=1}^{n} \left(\frac{\ln E}{X_i}\right) x_i$$

for all x_i's satisfying equation (7). The only solution is $\ln (f_i(x_i)) = \alpha_i x_i$ where $\alpha_i = (\ln E/X_i)$ (constant). Therefore $f_i(x_i) = \exp(\alpha_i x_i)$ that is, the effect of a zero-interactive combination is the product of the effects of its constituents only when all agents in the combination show exponential dose-effect relations.